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Wavelength-selective fluorescence in ion channels formed by gramicidin A in membranes

AMITABHA CHATTOPADHYAY* and SATINDER S RAWAT[#]
Centre for Cellular and Molecular Biology, Uppal Road, Hyderabad 500 007
[#]Present address: 364 Plantation Street, Room 570R, Lazare Research Building, University of Massachusetts Medical School, Worcester, MA 01605, USA
e-mail: amit@ccmb.res.in

Abstract. Gramicidins are linear peptides that form ion channels that are specific for monovalent cations in membranes. The tryptophan residues in the gramicidin channel play a crucial role in the organization and function of the channel. The natural mixture of gramicidins, denoted as gramicidin A′, consists of mostly gramicidin A, but also contains gramicidins B, C and D as minor components. We have previously shown that the tryptophan residues in ion channels formed by the naturally occurring peptide, gramicidin A′, display wavelength-dependent fluorescence characteristics due to the motionally restricted environment in which they are localized. In order to check the influence of ground-state heterogeneity in the observed wavelength-selective fluorescence of gramicidin A′ in membranes, we performed similar experiments with pure gramicidin A in model membranes. Our results show that the observed wavelength-selective fluorescence characteristics of naturally occurring gramicidin A′ are not due to ground-state heterogeneity.

Keywords. Gramicidins; ion channels; wavelength-dependent fluorescence; wavelength-selective fluorescence; red-edge excitation shift.

1. Introduction

The linear peptide gramicidin forms prototypical ion channels that are specific for monovalent cations and has been extensively used to study the organization, dynamics and function of membrane-spanning channels.[1–3] The transmembrane gramicidin channel is formed by the head-to-head dimerization of $b^{6.3}$ helices.[4] The channel interior is lined by the polar carbonyl and amide moieties of the peptide backbone, a feature shared with the selectivity filter of the bacterial KcsA K^+ channel.[5] An important aspect of this conformation is the membrane interfacial location of the tryptophan residues, a common feature of many transmembrane helices.[6–8]

Gramicidins are linear pentadecapeptide antibiotics with a molecular weight of ~1900. They are produced by the soil bacterium *Bacillus brevis*, and consist of alternating L- and D-amino acids.[9] The natural mixture of gramicidins, often denoted as gramicidin A′ (termed as gramicidin D in older literature), consists of gramicidin A (80–85%), gramicidin B (6–7%), gramicidin C (5–14%), and gramicidin D (1%).[10] The various types of gramicidins differ in one residue. Gramicidin A and D have four tryptophan residues at positions 9, 11, 13 and 15 (see table 1). However, the Trp-11 in gramicidin A and D is replaced by Phe in gramicidin B, and Tyr in gramicidin C. On the other hand, the Gly-2 of gramicidin A is replaced by Ala in gramicidin D. Gramicidin A′ is readily available commercially and is fluorescent, due to the presence of tryptophan residues.[11,12] It has one of the most hydrophobic sequences known and has been widely used as a model peptide for membrane-spanning regions of intrinsic membrane proteins.[13,14]

The tryptophan residues in gramicidin channels are believed to be crucial for maintaining the structure and function of the channel.[15–17] We have earlier performed wavelength-selective fluorescence experiments using the tryptophan residues in the channel conformation of the naturally occurring peptide, gramicidin A′.[11,18] Wavelength-selective fluorescence comprises a set of approaches based on the red-edge effect in fluorescence spectroscopy, which can be used to directly monitor the environment and dynamics around a fluorophore in a complex biological system. A shift in the wavelength of maximum fluorescence

*For correspondence

Table 1. Amino acid sequences of various gramicidin peptides. The differences in sequence among the peptides and positions of the tryptophan residues are highlighted.

Gramicidin type	Amino acid sequence	Percent content in gramicidin A′
A	HCO-L-Val-Gly-L-Ala-D-Leu-L-Ala-D-Val-L-Val-D-Val-L-Trp-D-Leu-L-Trp-D-Leu-L-Trp-D-Leu-L-Trp-NHCH$_2$CH$_2$OH 9 11 13 15	80–85%
B	HCO-L-Val-Gly-L-Ala-D-Leu-L-Ala-D-Val-L-Val-D-Val-L-Trp-D-Leu-L-Phe-D-Leu-L-Trp-D-Leu-L-Trp-NHCH$_2$CH$_2$OH 9 11 13 15	6–7%
C	HCO-L-Val-Gly-L-Ala-D-Leu-L-Ala-D-Val-L-Val-D-Val-L-Trp-D-Leu-L-Tyr-D-Leu-L-Trp-D-Leu-L-Trp-NHCH$_2$CH$_2$OH 9 11 13 15	5–14%
D	HCO-L-Va-D-Ala-L-Ala-D-Leu-L-Ala-D-Val-L-Val-D-Val-L-Trp-D-Leu-L-Trp-D-Leu-L-Trp-D-Leu-L-Trp-NHCH$_2$CH$_2$OH 9 11 13 15	1%

emission toward higher wavelengths, caused by a shift in the excitation wavelength toward the red-edge of the absorption band, is termed red edge excitation shift (REES). This effect is mostly observed with polar fluorophores in motionally restricted media such as very viscous solutions or condensed phases, where the dipolar relaxation time for the solvent shell around a fluorophore is comparable to or longer than its fluorescence lifetime.[19–23] REES arises from slow rates of solvent relaxation (reorientation) around an excited-state fluorophore which depends on the motional restriction imposed on the solvent molecules in the immediate vicinity of the fluorophore. Utilizing this approach, it becomes possible to probe the mobility parameters of the environment itself (which is represented by the relaxing solvent molecules) using the fluorophore merely as a reporter group. Further, since the ubiquitous solvent for biological systems is water, the information obtained in such cases would come from the otherwise 'optically silent' water molecules.

We have previously shown that the tryptophan residues in channels formed by the naturally occurring peptide, gramicidin A′, exhibit REES due to the motionally restricted environment in which they are localized in this conformation in the membrane interface.[11,18] Since gramicidin A′ is not a chemically pure compound and is a mixture of peptides with some peptides (gramicidin B and C) having different number of tryptophan residues, the observed REES from channels formed by gramicidin A′ could be due to ground-state heterogeneity. In this paper, we have addressed this issue by performing wavelength-selective fluorescence experiments on the channel conformation formed by pure gramicidin A in model membranes.

2. Materials and methods

2.1 *Materials*

1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) was obtained from Avanti Polar Lipids (Alabaster, AL, USA). Pure gramicidin A was purified from commercial gramicidin A′ (from *Bacillus brevis*) and was a generous gift from Prof. Roger E Koeppe (University of Arkansas, USA). Dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Lipids were checked for purity by thin layer chromatography on silica gel precoated plates (Sigma) in chloroform/methanol/water (65 : 35 : 5, v/v/v) and were found to give only one spot in all cases with a phosphate-sensitive spray and subsequent charring.[24] The concentration of POPC was determined by phosphate assay subsequent to total digestion by perchloric acid.[25] DMPC was used as an internal standard to assess lipid digestion. All other chemicals used were of the highest purity available. Solvents used were of spectroscopic grade. Water was purified through a Millipore (Bedford, MA, USA) Milli-Q system and used for all experiments.

2.2 *Sample preparation*

All experiments were done using unilamellar vesicles (ULV) of POPC containing 2% (mol/mol) gramicidin A. The channel conformation of gramicidin A was generated essentially as described earlier.[11] In general, 1280 nmol of POPC in chloroform was mixed with 25·6 nmol of gramicidin A in methanol. A few drops of chloroform were added and mixed well, and the samples were dried under a stream of nitrogen while being warmed gently (~35°C). After further drying under high vacuum for at least 3 h, 3 ml of 10 mM sodium phosphate, and 150 mM of sodium chloride buffer, pH 7·0–7·2, was added, and each sample vortexed for 3 min to disperse the lipid. The lipid dispersions so obtained were sonicated for 10 min (in bursts of 1 min, followed by immediate cooling in ice) using a Branson model 250 sonifier fitted with a microtip. The sonicated samples were centrifuged at 15,000 rpm for 20 min to remove titanium particles shed from the microtip during sonication and incubated overnight at 65°C with continuous shaking in order to induce channel conformation.[26,27] Background samples were prepared the same way except that gramicidin was omitted. All experiments were done with multiple sets of samples at 25°C.

2.3 *Steady state fluorescence measurements*

Steady state fluorescence measurements were performed with a Hitachi F-4010 steady state spectrofluorometer using 1-cm path length quartz cuvettes. Excitation and emission slits with a nominal bandpass of 5 nm were used. Background intensities of samples in which gramicidin A was omitted were subtracted from each sample spectrum to cancel out any contribution due to the solvent Raman peak and other scattering artifacts. The spectral shifts obtained with different sets of samples were identical in most cases. In other cases, the values were within ± 1 nm of the ones reported. Fluorescence polarization measurements were performed using a Hitachi polarization accessory. Polarization values were calculated from the equation,[28]

$$P = (I_{VV} - GI_{VH})/(I_{VV} + GI_{VH}), \quad (1)$$

where I_{VV} and I_{VH} are the measured fluorescence intensities (after appropriate background subtraction) with the excitation polarizer vertically oriented and emission polarizer vertically and horizontally oriented, respectively. G is the grating correction factor and is the ratio of the efficiencies of the detection system for vertically and horizontally polarized light, and is equal to I_{HV}/I_{HH}. All experiments were done with multiple sets of samples and average values of polarization are shown in figures 3 and 4.

2.4 *Circular dichroism measurements*

CD measurements were carried out at room temperature (25°C) on a Jasco J-715 spectropolarimeter which was calibrated with (+)-10-camphorsulphonic acid.[29] The spectra were scanned in a quartz optical cell with a path length of 0·1 cm. All spectra were recorded in 0·5 nm wavelength increments with a 4 s response and a band width of 1 nm. For monitoring changes in secondary structure, spectra were scanned in the far-UV range from 200 to 280 nm at a scan rate of 100 nm/min. Each spectrum is the average of 12 scans with full-scale sensitivity of 10 mdeg. All spectra were corrected for background by subtraction of appropriate blanks and were smoothed making sure that the overall shape of the spectrum remains unaltered. Data are represented as mean residue ellipticities and were calculated using the formula:

$$[q] = q_{obs}/(10Cl), \quad (2)$$

where q_{obs} is the observed ellipticity in mdeg, l is the path length in cm, and C is the concentration of peptide bonds in mol/L.

3. Results and discussion

3.1 *Circular dichroism spectroscopy shows that gramicidin A forms channels in membranes*

Circular dichroism spectroscopy has been previously utilized to characterize various membrane-bound conformations of gramicidin.[26,27] We therefore used characteristic CD spectroscopic features to confirm the conformation of gramicidin A in POPC vesicles. The CD spectrum of gramicidin A in POPC vesicles is shown in figure 1. The spectrum for the channel conformation of gramicidin has two characteristic peaks of positive ellipticity around 218 and 235 nm, a valley around 230 nm, and negative ellipticity below 208 nm.[26,27] These are characteristic of the single-stranded $b^{6\cdot3}$ conformation. As shown in figure 1, the CD spectrum of gramicidin A agrees with this criterion, and is therefore representative of the channel conformation.

3.2 *Red-edge excitation shift of gramicidin A tryptophans*

Shift in the maxima of fluorescence emission[‡] of the tryptophan residues of gramicidin A in the channel conformation as a function of excitation wavelength is shown in figure 2. Tryptophans in the channel form of gramicidin A exhibit an emission maximum of 332 nm, when excited at 280 nm, similar to that previously reported for gramicidin A′.[11,18] As the excitation wavelength is changed from 280 to 310 nm, the emission maximum is shifted from 332 to 338 nm, which corresponds to a REES of 6 nm. It is possible that there could be further red shift if excitation is carried out beyond 310 nm. We found it difficult to work in this wavelength range due to low signal-to-noise ratio and artifacts due to the solvent Raman peak that sometimes remained even after background subtraction.

Such dependence of the emission maximum on excitation wavelength is characteristic of the red-edge excitation shift. This implies that the tryptophans in the channel conformation of gramicidin A are localized in a motionally restricted region of the membrane. This is consistent with our earlier findings that the channel tryptophans gramicidin A′ are localized at the membrane interface.[11,18] Interestingly, the REES exhibited by channels formed by gramicidin A′[11,18] and gramicidin A (present results) were found to be essentially the same. This rules out any contribution of ground-state heterogeneity toward the observed REES of gramicidin A′.

3.3 *Wavelength-dependent fluorescence polarization of gramicidin A tryptophans*

Fluorescence polarization is known to be dependent on excitation and emission wavelengths in motionally restricted media.[21] The excitation polarization spectrum (i.e., a plot of steady-state polarization vs. excitation wavelength) of gramicidin A in POPC vesicles is shown in figure 3. The fluorescence polarization of gramicidin A in the membrane displays characteristic change upon increasing the excitation wavelength, with a sharp increase occurring toward the red-edge of the absorption band. Such an increase in polarization upon red-edge excitation for peptides

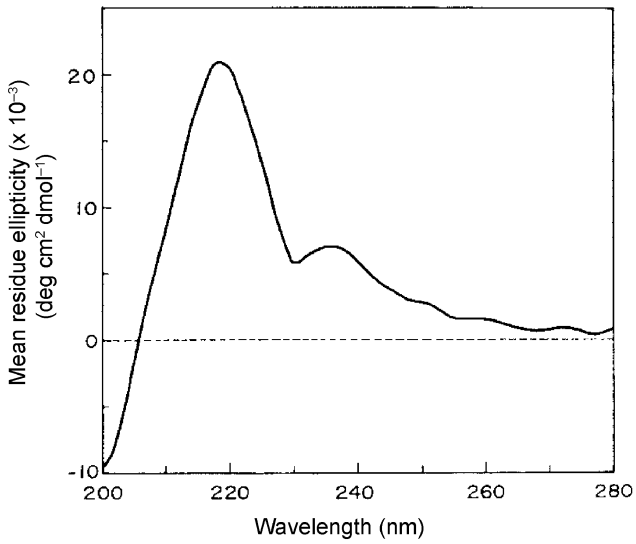

Figure 1. Far-UV CD spectra of the channel form of gramicidin A in vesicles of POPC. The ratio of gramicidin to POPC was 1 : 50 (mol/mol). See §2 for other details.

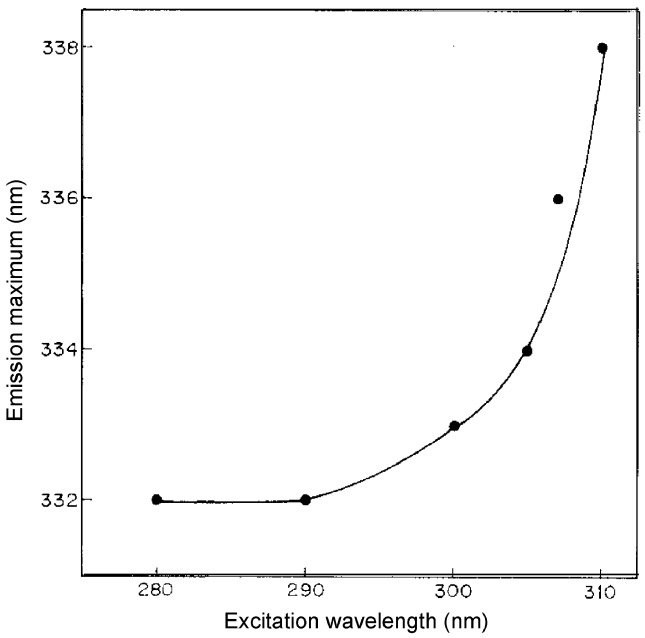

Figure 2. Effect of changing excitation wavelength on the wavelength of maximum emission for the channel form of gramicidin A. The ratio of gramicidin to POPC was 1 : 50 (mol/mol). All other conditions are as in figure 1. See §2 for other details.

[‡]We have used the term maximum of fluorescence emission in a somewhat wider sense here. In every case, we have monitored the wavelength corresponding to maximum fluorescence intensity, as well as the centre of mass of the fluorescence emission. In most cases, both these methods yielded the same wavelength. In cases where minor discrepancies were found, the centre of mass of emission has been reported as the fluorescence maximum.

and proteins containing tryptophans in media of reduced mobility has been reported before.[21] This reinforces that the tryptophan residues in gramicidin A are localized in a motionally restricted region of the membrane. As a control, the fluorescence polarization of gramicidin A in methanol was monitored, which remained essentially invariant over the range of excitation wavelengths. Figure 4 shows the variation in steady-state polarization of tryptophan residues of gramicidin A in POPC vesicles and in methanol, as a function of wavelength across its emission spectrum. As seen from the figure, while polarization values do not show any significant variation over the entire emission range in methanol, there is a considerable decrease in polarization with increasing emission wavelength in case of membrane-bound gramicidin A, as expected for fluorophores in a restricted environmernt.[21] These wavelength-dependent changes in fluorescence polarization of gramicidin A tryptophans are similar to the changes previously observed with membrane-bound gramicidin A′.[11]

Although the functional properties (channel conductance) displayed by gramicidins A, B, and C are different, the backbone conformations of these peptides in micelles have previously been found to be essentially identical using NMR spectroscopy.[30] Interestingly, it has earlier been reported that fluorescence lifetimes of pure gramicidin A in model membranes are identical with those obtained for the commercially available gramicidin A′ samples.[31] In addition, ground state heterogeneity arising either due to the presence of gramicidin A in the bulk aqueous phase or due to the presence of gramicidin A monomers in the membrane can be ruled out as follows. Contribution from gramicidin A monomer in water is unlikely since gramicidin A is very hydrophobic resulting in extremely low solubility in aqueous solutions ($\approx 5 \times 10^{-7}$ M).[32] Also, the dimerization constant of gramicidin A in DOPC black lipid membranes to be $\approx 2 \times 10^{13}$ mol^{-1} cm^2.[33] This means that at the peptide concentration generally employed for spectroscopic experiments in lipid vesicles, the fraction of monomers is negligible. Taken together, our results show that the observed wavelength-selective fluorescence characteristics of naturally occurring gramicidin A′, which is a mixture of peptides, is not due to ground-state heterogeneity, since essentially the similar results are obtained when similar

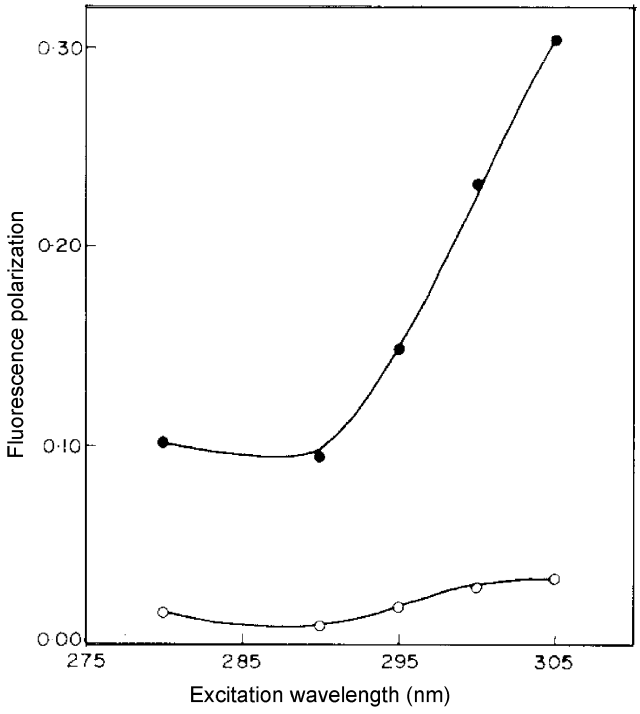

Figure 3. Fluorescence polarization of the channel form of gramicidin A in POPC vesicles (●) as a function of excitation wavelength. Fluorescence polarization of gramicidin A in methanol (○) as a function of excitation wavelength is shown as a control. Polarization values were recorded at 331 nm. The ratio of gramicidin to POPC was 1 : 50 (mol/mol). Concentration of gramicidin A in methanol was 8·6 *m*M. See §2 for other details.

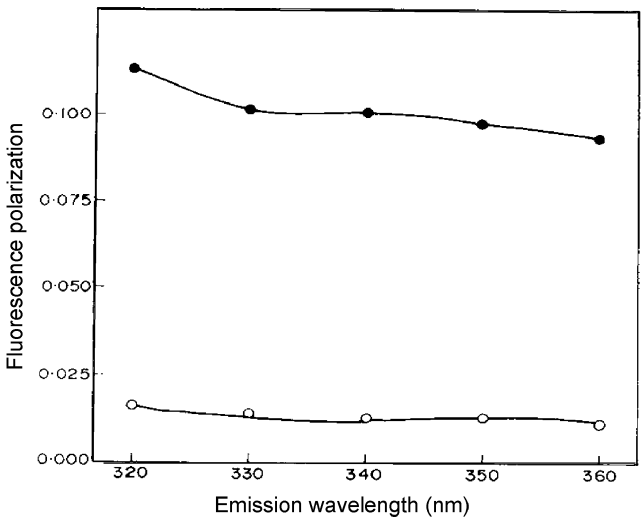

Figure 4. Fluorescence polarization of the channel form of gramicidin A in POPC vesicles (●) as a function of emission wavelength. Fluorescence polarization of gramicidin A in methanol (○) as a function of emission wavelength is shown as a control. The excitation wavelength was 280 nm. The ratio of gramicidin to POPC was 1 : 50 (mol/mol). Concentration of gramicidin A in methanol was 8·6 *m*M. See §2 for other details.

experiments were performed with chemically pure gramicidin A.

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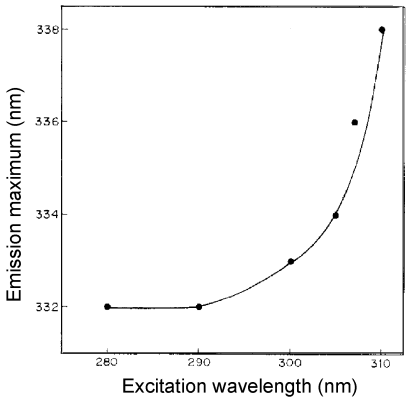
References

1. Killian J A 1992 *Biochim. Biophys. Acta* **1113** 391
2. Koeppe R E and Andersen O S 1996 *Annu. Rev. Biophys. Biomol. Struct.* **25** 231
3. Wallace B A 2000 *BioEssays* **22** 227
4. O'Connell A M, Koeppe R E and Andersen O S 1990 *Science* **250** 1256
5. Kelkar D A and Chattopadhyay A 2006 *J. Biosci.* **31** 297
6. Reithmeier R A F 1995 *Curr. Opin. Struct. Biol.* **5** 491
7. Yau W-M, Wimley W C, Gawrisch K and White S H 1998 *Biochemistry* **37** 14713
8. Ulmschneider M B and Sansom M S P 2001 *Biochim. Biophys. Acta* **1512** 1
9. Sarges R and Witkop B 1965 *J. Am. Chem. Soc.* **87** 2011
10. Abo-Riziq A, Crews B O, Callahan M P, Grace L and de Vries M S 2006 *Angew. Chem., Int. Ed.* **45** 5166
11. Mukherjee S and Chattopadhyay A 1994 *Biochemistry* **33** 5089
12. Rawat S S, Kelkar D A and Chattopadhyay A 2005 *Biophys. J.* **89** 3049
13. Andersen O S, Nielsen C, Maer A M, Lundbæk J A, Goulian M and Koeppe R E 1999 *Methods Enzymol.* **294** 208
14. Martinac B and Hamill O P 2002 *Proc. Natl. Acad. Sci. USA* **99** 4308
15. Hu W, Lee K-C and Cross T A 1993 *Biochemistry* **32** 7035
16. Salom D, Pérez-Payá E, Pascal J and Abad C 1998 *Biochemistry* **37** 14279
17. Andersen O S, Greathouse D V, Providence L L, Becker M D and Koeppe R E 1998 *J. Am. Chem. Soc.* **120** 5142
18. Rawat S S, Kelkar D A and Chattopadhyay A 2004 *Biophys. J.* **87** 831
19. Demchenko A P 1988 *Trends Biochem. Sci.* **13** 374
20. Demchenko A P 2002 *Luminescence* **17** 19
21. Mukherjee S and Chattopadhyay A 1995 *J. Fluoresc.* **5** 237
22. Chattopadhyay A 2003 *Chem. Phys. Lipids* **122** 3
23. Raghuraman H, Kelkar D A and Chattopadhyay A 2005 *Reviews in fluorescence* vol 2 (eds) C D Geddes and J R Lakowicz (Springer: New York) 199–222
24. Dittmer J C and Lester R L 1964 *J. Lipid Res.* **5** 126
25. McClare C W F 1971 *Anal. Biochem.* **39** 527
26. Killian J A, Prasad K U, Hains D and Urry D W 1988 *Biochemistry* **27** 4848
27. LoGrasso P V, Moll F and Cross T A 1988 *Biophys. J.* **54** 259
28. Lakowicz J R 2006 *Principles of fluorescence spectroscopy* (New York: Springer)
29. Chen G C and Yang J T 1977 *Anal. Lett.* **10** 1195
30. Townsley L E, Tucker W A, Sham S and Hinton J F 2001 *Biochemistry* **40** 11676
31. Camaleno-Delango J-M, Zhao X K and Fendler J H 1990 *Can. J. Chem.* **68** 888
32. Kemp G, Jacobson K A and Wenner C E 1972 *Biochim. Biophys. Acta* **255** 493
33. Veatch W R, Mathies R, Eisenberg M and Stryer L 1975 *J. Mol. Biol.* **99** 75

Graphical Abstract (PC-MPSI-10)



Wavelength-selective fluorescence in ion channels formed by gramicidin A in membranes

Amitabha Chattopadhyay and Satinder S Rawat **00–00**

Wavelength-selective fluorescence of pure gramicidin A in membranes is similar to that of gramicidin A′, the natural mixture of gramicidins, which consists mostly of gramicidin A, but also has other gramicidin variants as minor components. Our results show that the observed wavelength-selective fluorescence characteristics of the naturally occurring gramicidin A' are not due to ground-state heterogeneity.